Host responses in the bursa of Fabricius of chickens infected with virulent Marek's disease virus

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ABSTRACT

The bursa of Fabricius serves as an important tissue in the process of Marek's disease virus (MDV) pathogenesis, since B cells of the bursa harbor the cytolytic phase of MDV replication cycle. In the present study, host responses associated with MDV infection in the bursa of Fabricius of chickens were investigated. The expression of MDV phosphoprotein (pp)38 antigen, MDV glycoprotein (gB) and MDV viral interleukin (vIL)-8 transcripts was at the highest at 4 days post-infection (d.p.i.) and then showed a declining trend. On the contrary, the expression of meq (MDV EcoRI Q) gene as well as the viral genome load increased gradually until day 14 post-infection. The changes in viral parameters were associated with significantly higher infiltration of macrophages and T cell subsets, particularly CD4+ T cells into the bursa of Fabricius. Of the genes examined, the expression of interferon (IFN)-α, IFN-γ genes and inducible nitric oxide synthase (iNOS) was significantly up-regulated in response to MDV infection in the bursa of Fabricius. The results suggest a role for these cells and cytokines in MDV-induced responses in the bursa of Fabricius.

Introduction

Marek's disease virus (MDV) is an alphaherpesvirus, which belongs to the genus *Mardivirus* (Davison et al., 2002). MDV infection in chickens leads to the formation of T cell tumors in various body tissues, neurological manifestations as well as immune suppression (Calnek, 2001; Payne, 2004). Feather dander and poultry house dust serve as the source for inhalation of infectious MDV and subsequent establishment of natural infection (Beasley et al., 1970; Calnek et al., 1970). Initial respiratory infection is followed by the cytolytic phase that extends from 3–6 days post-infection (d.p.i.) and occurs in lymphoid organs such as spleen, the bursa of Fabricius and thymus (Calnek, 2001). MDV established in the respiratory system is carried to other organs by infected macrophages (Barrow et al., 2003). In lymphoid organs, mainly B cells are infected initially by MDV (Shek et al., 1983; Calnek et al., 1984; Baigent et al., 1998). The importance of the bursa of Fabricius for the successful completion of MDV replication cycle has been shown (Schat et al., 1981). The absence of this phase, leads to lower viral replication resulting in lower viraemia and decreased incidence of tumor formation (Schat et al., 1981). Following a burst of productive/restrictive infection in B cells that is associated with high transcriptional activity of MDV phosphoprotein (pp)38 antigen (Baigent et al., 1998; Burgess et al., 2001; Burgess and Davison, 2002), a switch to latent infection in T cells occurs approximately 7 d.p.i. The

switching of infection may be influenced by a protein encoded by MDV, viral interleukin (vIL)-8 that acts as a chemoattractant for chicken T cells (Liu et al., 1999; Parcells et al., 2001) and allows the infiltration of T cells to the vicinity of MDV-infected B cells. Alternatively, MDV-infected cells can up-regulate major histocompatibility complex (MHC) class II molecules on MDV-infected cells (Niikura et al., 2007) which may facilitate presentation of MDV antigens (Malnati et al., 1992) and initiation of host response, allowing T cell infiltration into the site of virus replication. MDV infection in T cells becomes latent probably due to host responses elicited by the virus (Buscaglia et al., 1988; Volpini et al., 1996). The latent phase is followed by transformation of T cells and tumor formation in Marek's disease (MD) susceptible chickens (Calnek, 2001).

Host responses elicited against MDV infection have been studied in spleen, which serves as a major secondary lymphoid organ in the chicken and harbors almost all the stages of the MDV replication cycle (Xing and Schat, 2000a; Kaiser et al., 2003; Jarosinski et al., 2005; Sarson et al., 2006). Recently, studies have been focused on elucidating host responses at the site of virus shedding (Abdul-Careem et al., 2008), in the central nervous system (Gimeno et al., 2001; Jarosinski et al., 2005; Abdul-Careem et al., 2006b) and also in blood (Quere et al., 2005), since all these body compartments are engaged in different phases of the MDV replication cycle. The cytolytic phase is an important phase of the MDV replication cycle that mainly involves the bursa of Fabricius (Schat et al., 1981) and the consequence of this phase is usually immune suppression leading to increased susceptibility to other secondary infections (Islam et al.,
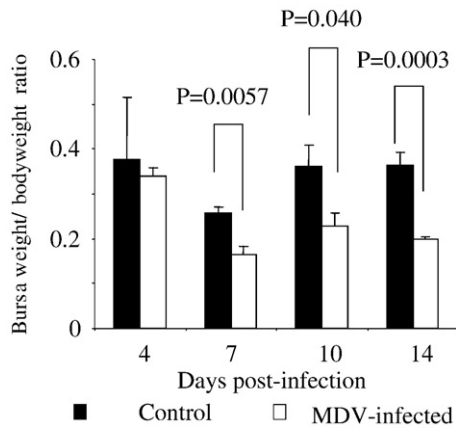
Fig. 1. Bursa/bodyweight ratios of chickens infected with RB1B strain of MDV and uninfected controls. The groups were as follows: MDV-infected=chickens that were infected with MDV and sampled at 4, 7, 10 and 14 d.p.i. and Control=age-matched chickens that were not infected. There were four chickens in each group at each time point. Differences between groups were assessed by student t test and comparisons were considered significant at $P \leq 0.05$.

2002). Although, cell-mediated immune responses as characterized by the infiltration of T cells and macrophages (Gimeno et al., 2001; Abdul-Careem et al., 2008) and expression of cytokine genes (Xing and Schat, 2000a; Kaiser et al., 2003; Jarosinski et al., 2005; Quere et al., 2005; Abdul-Careem et al., 2006b, 2008) have been investigated in various tissues, there is no information available on host responses in the bursa of Fabricius

following MDV infection. This is an important lymphoid organ, which perform both primary as well as secondary immune functions.

Host responses characterized by cell infiltration and expression of cytokine genes have been studied in the bursa of Fabricius in response to infectious bursal disease virus (IBDV) infection in chickens (Tanimura and Sharma, 1997; Kim et al., 1999, 2000; Rautenschlein et al., 2002a; Khatri et al., 2005; Eldaghayes et al., 2006; Palmquist et al., 2006). These responses, especially T cell responses, have been shown to be effective in controlling IBDV replication (Kim et al., 2000; Rautenschlein et al., 2002a,b). In terms of cytokine responses, high transcriptional activities of interferon (IFN)-γ (Kim et al., 2000; Rautenschlein et al., 2003; Eldaghayes et al., 2006), IFN-β (Eldaghayes et al., 2006) and proinflammatory cytokines such as IL-18 (Khatri et al., 2005; Palmquist et al., 2006) and IL-6 (Kim et al., 2000; Eldaghayes et al., 2006; Khatri et al., 2005; Palmquist et al., 2006) have been shown in the bursa of Fabricius in response to IBDV infection.

Given the paucity of information on the mechanisms of host response to MDV interaction in the bursa of Fabricius, which carries an important phase of the MDV life cycle, the objective of the present study was to investigate the cellular and cytokine responses in this lymphoid organ subsequent to MDV infection.

Results

Weight of the bursa of Fabricius of MDV-infected and -uninfected chickens

Bursa weight as a percentage of bodyweight of MDV-infected and uninfected control chickens are illustrated in Fig. 1. The bursa/
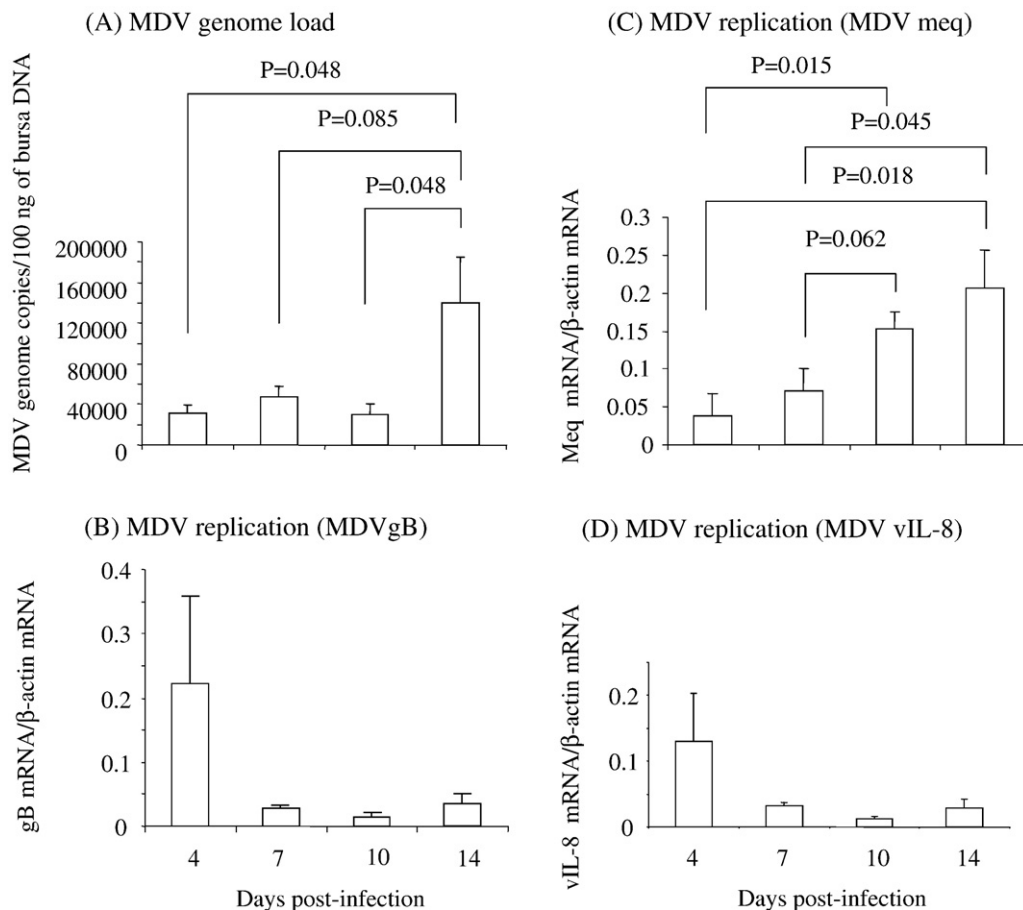

Fig. 2. MDV genome load and MDV transcripts in bursa of Fabricius of chickens infected with RB1B strain of MDV. Chickens were infected with MDV and sampled at 4, 7, 10 and 14 d.p.i. There were five MDV-infected chickens at each time point. Mean MDV genome load (A), gB mRNA (B), meq mRNA (C) and vIL-8 mRNA (D) expression relative to β-actin mRNA expression are presented and the error bars represent standard error of the mean.

bodyweight ratio in MDV-infected chickens was significantly lower compared to the controls at 7 (P=0.0057), 10 (P=0.040) and 14 (P=0.0003) d.p.i.

MDV Genome load in the bursa of Fabricius of MDV-infected chickens

MDV genome load was quantified in the bursa, which harbors the cytolytic phase of the virus replication cycle. Initial screening of the bursa DNA by conventional PCR determined that uninfected controls had remained MDV free, whereas the meq gene could be amplified from all tested DNA samples derived from MDV-infected chickens. Bursa DNA originated from MDV-infected chickens was further analyzed by real-time PCR and the data are illustrated in Fig. 2(A). MDV genome was quantifiable from all the observed time points. MDV-infected chickens had 31470±17161, 47816±21815, 30531± 22480 and 139339±101964 MDV genome per 100 ng of tissue DNA
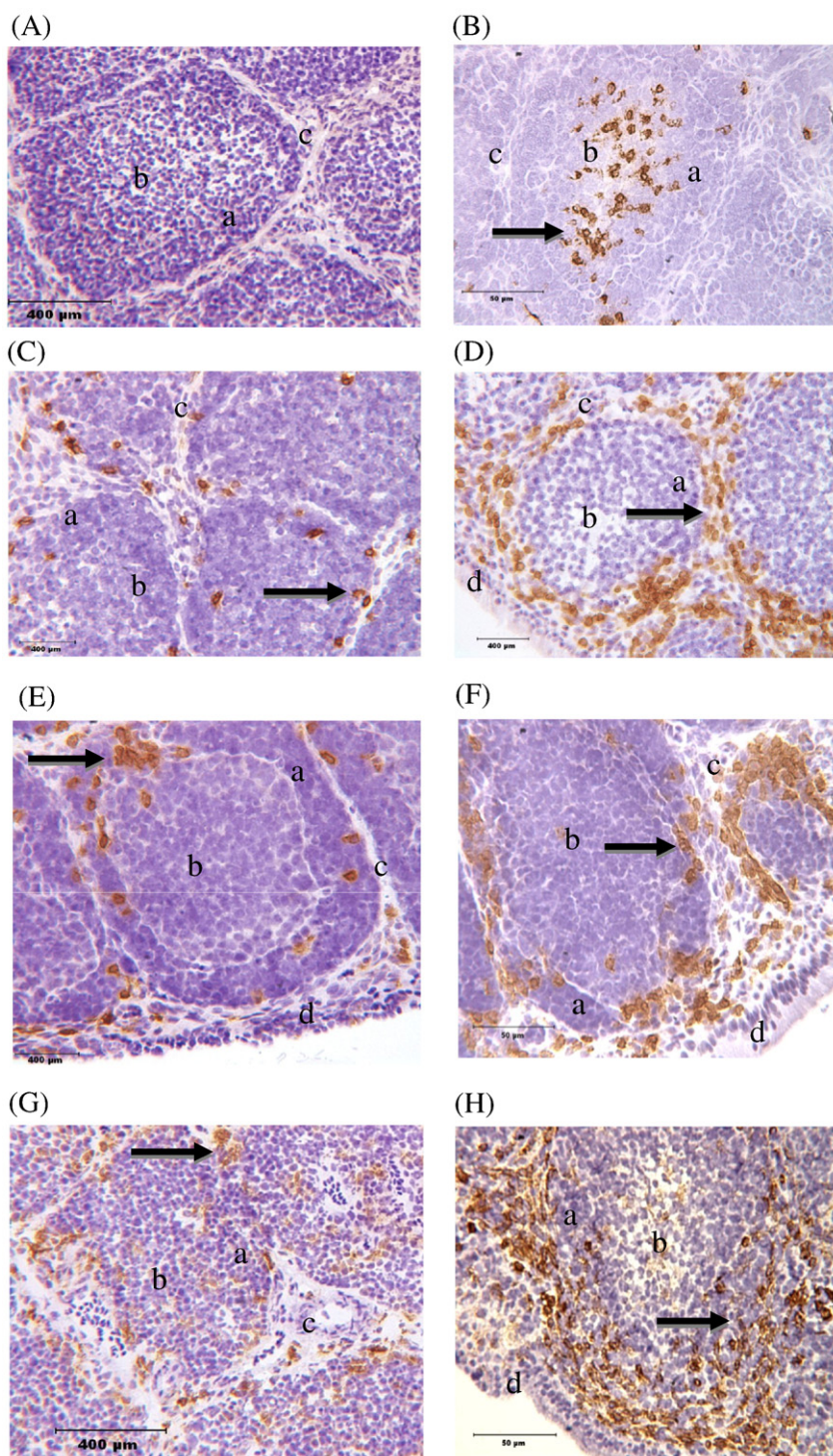

Fig. 3. Immunohistochemistry analysis of the bursa of Fabricius of chickens infected with RB1B strain of MDV on day 4 post-infection. Bursa tissues of uninfected controls are shown in A, C, E, G, whereas B, D, F, H represent bursa sections from infected chickens. A, B = anti-pp38 staining, C, D = anti-CD4 staining, E, F = anti-CD8 staining, G, H = staining for macrophages. Bar = 400 μm (50 μm in C, G and H). Arrows show positively stained cells. a = cortex of a bursal follicle, b = medulla of a bursal follicle, c = inter-follicular area, d = follicular epithelium.

at 4, 7, 10 and 14 d.p.i., respectively. The MDV genome load observed at 14 d.p.i. was significantly higher when compared to that observed at 4, 7 and 10 d.p.i. (P≤0.05).

MDV replication in the bursa of Fabricius of MDV-infected chickens

MDV replication was assessed by quantification of transcripts of MDV gB, meq and vIL-8 genes, which are associated with different phases of the MDV replication cycle. MDV-infected chickens that were sampled at 4 d.p.i. had more gB transcripts in their bursa compared to MDV-infected chickens that were sampled throughout the rest of the study (Fig. 2B). However, the differences between observations were not significant (P>0.05). The pattern of MDV meq transcripts was not comparable to that observed for gB transcripts (Fig. 2C). The expression of the meq gene at 4 d.p.i. was significantly lower than that observed on 10 and 14 d.p.i. (P=0.015 and 0.018 respectively). Similarly, the expression of the meq gene at 7 d.p.i. was marginally and significantly lower than that observed on days 10 and 14 post-inoculation (P=0.062 and 0.045, respectively). The expression of vIL-8 gene in the bursa followed the expression pattern of gB gene, being higher at 4 d.p.i., followed by a decline at 7 d.p.i. and maintaining a low expression level for the rest of study period (Fig. 2D). However, the differences between observations were not significant (P>0.05).

Histological observations

Infiltration of cells, such as MDV pp38+ cells, CD4+ and CD8+ T cells and macrophages in response to MDV infection was quantified following immunohistochemical staining of the bursa sections. Infiltration of CD4+, CD8+ T cells and macrophages was observed in the bursa samples of both MDV-infected and control chickens at 4, 7, 10 and 14 d.p.i. The cells that expressed MDV pp38+ antigen were observed mainly in the medulla of bursal follicles of infected birds (Fig. 3B). The infiltration of CD4+ and CD8+ T cells and macrophages, however, were observed mainly in the inter-follicular areas and to a lesser degree in the cortex and medulla of follicles (Figs. 3C–H).

The mean number of cells that expressed the pp38 antigen was higher on 4 d.p.i. Subsequently, the number of pp38+ cells declined at 7 d.p.i., but then slightly increased towards the end of the study (Fig. 4A), although the differences between groups were not significant (P>0.05). Increased infiltration of macrophages and T cell subsets in the vicinity of follicles containing cells that express pp38 antigen was noticeable, particularly in tissue sections of MDV-infected chickens sampled at 4 d.p.i. This relationship between viral antigen expressing cells and cellular response was not evident in subsequent sampling occasions due to a substantial decrease in the number of pp38+ cells.

Infiltration of CD4+ T cells was higher in the bursa of MDV-infected chickens when compared to the controls. Infiltration of CD4+ T cells in the bursa of MDV-infected chickens sampled on days 7 and 10 post infection was marginally (P=0.0962) and significantly (P=0.0098) higher, respectively, when compared to the age-matched controls (Fig. 4B). Infiltration of CD4+ T cells in the bursa of MDV-infected chickens sampled at 10 d.p.i. was significantly higher when compared to the MDV-infected chickens sampled at 4 d.p.i. (P=0.0038).
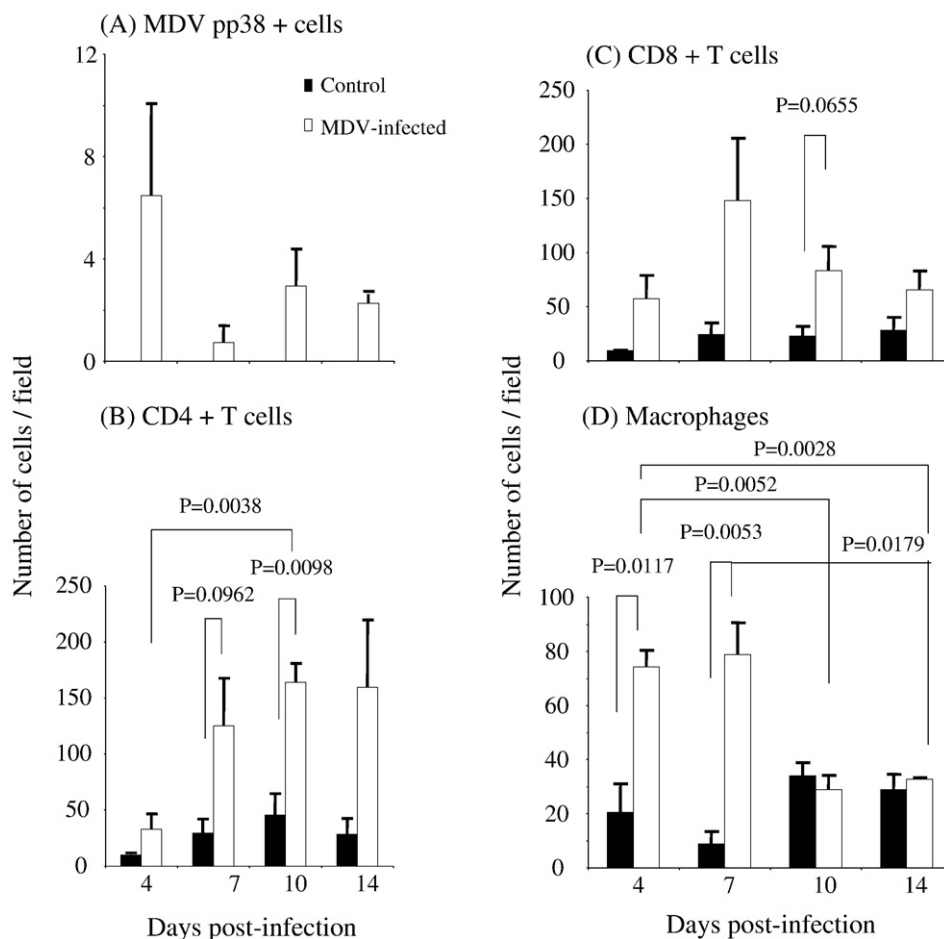

Fig. 4. Distribution of MDV pp38 + cells, T cell subsets and macrophages in the bursa of Fabricius of chickens infected with RB1B strain of MDV. The groups were as follows: MDV-infected = chickens that were infected with MDV and sampled at 4, 7, 10 and 14 d.p.i. and Control = age-matched chickens that were not infected. There were three chickens in each group at each time point. Group mean number of MDV pp38 + (A), CD4+ (B), CD8+ T (C) macrophage (D) cells per X40 microscopic field are presented and the error bars represent standard error of the mean.

Though the infiltration of CD8+ T cells was higher at all time points in the bursa of MDV-infected chickens when compared to the controls, only at 10 d.p.i. did this difference approach significance ($P=0.065$) (Fig. 4C).

Unlike the infiltration of T cells, macrophages were significantly higher on days 4 ($P=0.012$) and 7 ($P=0.0053$) post-inoculation in the bursa of MDV-infected chickens compared to the uninfected controls (Fig. 4D). Compared to macrophage counts recorded at 4 and 7 d.p.i., the macrophage counts observed at 10 ($P=0.018$ and 0.0052 respectively) and 14 d.p.i. ($P=0.018$ and 0.0028 respectively) were significantly lower.

Cytokine and iNOS gene expression in the bursa of Fabricius of MDV-infected and -uninfected chickens

The expression of IL-10 gene (Fig. 5A) was quantifiable in the bursa of both MDV-infected and uninfected control chickens. In response to MDV infection, there was an increase in IL-10 gene expression particularly at 4 and 7 d.p.i. On day 7 post-infection, the expression of IL-10 gene was marginally higher in the bursa of MDV-infected chickens when compared to uninfected controls ($P=0.084$). An age-related increasing trend in the expression of IL-10 gene was observed in the bursa of uninfected controls, although this was not statistically significant.

The expression of IL-18 gene was marginally higher in the bursa of MDV-infected chickens at 4 d.p.i. ($P=0.09$) (Fig. 5B). Similar to the expression of IL-10 gene, an age-related increase in the expression of IL-18 gene was observed in the bursa of uninfected controls.

In response to MDV infection, the mean expression of IFN-α in the bursa was the highest at 4 d.p.i., but its expression decreased at all other time points (Fig. 5C). The expression of IFN-α was significantly higher at 7 d.p.i. in the bursa of MDV-infected chickens compared to the controls ($P=0.013$). However, the expression of IFN-β gene was not significantly different in the bursa of infected compared to the

uninfected chickens ($P>0.05$) (Fig. 5D). The expression of IFN-γ gene was significantly higher at 4 ($P=0.02$) and 7 ($P=0.018$) d.p.i. in the bursa of infected chickens compared to that of the uninfected controls (Fig. 5E). However, this was followed by a sharp decline in the expression of IFN-γ gene in the bursa of infected chickens sampled at 10 d.p.i. compared to those collected at 7 d.p.i. ($P=0.0029$).

In response to MDV infection, the average expression of iNOS gene was higher in the bursa of MDV-infected chickens when compared to uninfected controls (Fig. 5F). The expression pattern of iNOS followed that of the IFN-γ gene. The expression of iNOS gene at 14 d.p.i. was significantly higher in the bursa of MDV-infected chickens compared to the age-matched uninfected controls ($P=0.0012$). A significant correlation (0.42) was observed between the expression of iNOS gene and infiltration of macrophages in the bursa of Fabricus ($P=0.041$).

Discussion

The bursa of Fabricius is a unique primary lymphoid organ, which plays an important role in B cell development and generation of the immunoglobulin repertoire (Cooper et al., 1966). The bursa of Fabricius may also act as a secondary lymphoid organ (Ekino et al., 1985). In the context of MDV infection, the bursa has been shown to provide an environment for a critical phase of MDV replication cycle, the cytolytic phase (Schat et al., 1981). The present study provides insight into the host immune responses, characterized by cellular activation and cytokine gene expression, to a very virulent RB1B strain of MDV in this organ. Analysis of viral parameters suggested that our observations are in agreement with the proposed MDV pathogenesis model (Calnek, 2001). As suggested, the cytolytic phase extends from 3–6 d.p.i. and the latent phase begins around 7–8 d.p.i. (Calnek, 2001). During the early cytolytic phase that occurs mainly in B cells (Shek et al., 1983; Calnek et al., 1984; Baigent et al., 1998), MDV predominantly expresses pp38, gB and vIL-8 genes (Baigent et al., 1998; Burgess et al.,
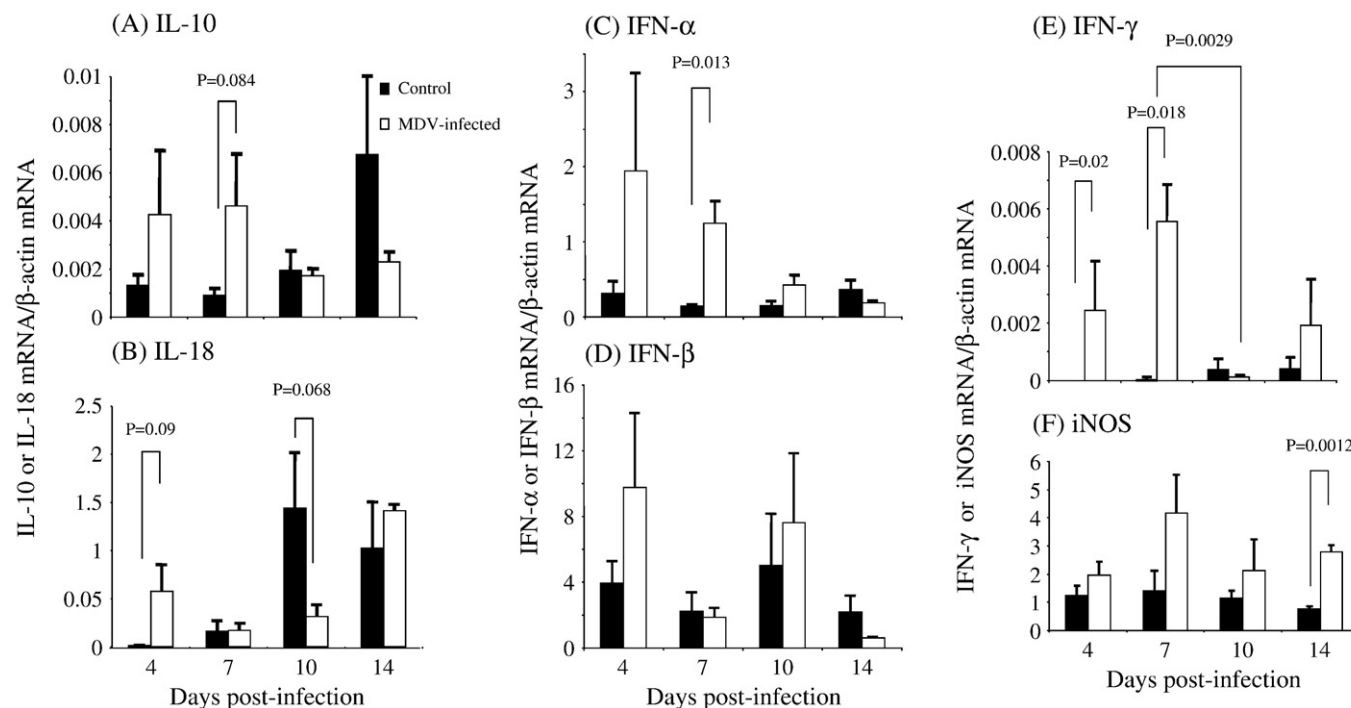

Fig. 5. Expression of cytokine genes in the bursa of Fabricius of chickens infected with RB1B strain of MDV. A–F show IL-10, IL-18, IFN-α, IFN-β, IFN-γ and iNOS mRNA expression, respectively. The groups were as follows: MDV-infected = chickens that were infected with MDV and sampled at 4, 7, 10 and 14 d.p.i. and Control = age-matched chickens that were not infected. There were five MDV-infected and 4 uninfected chickens at each time point. Target gene expression is presented relative to β-actin expression and normalized to a calibrator. Error bars represent standard error of the mean.

2001; Burgess and Davison, 2002; Cui et al. 2004). In the current study, this was demonstrated by the expression of gB and vIL-8 transcripts and pp38 antigen in the bursa of infected chickens. We discovered that MDV antigen pp38 was distributed mainly in the medulla of the infected follicles. Unlike the B cells in the cortex that are developing, B cells in the medulla of bursa follicles are more mature, IgM+ cells, which may ultimately be responsible for the generation of antibody-producing cells (Olah and Glick, 1992). Therefore, infection of these cells is partly responsible for immunosuppression caused by MDV.

Calnek et al. (1998) studied the potential of MDV pathotypes leading to immunosuppression using lymphoid organ weights including the bursa of Fabricius and found that the degree of immune suppression is related to the degree of virulence. In the present study, infected chickens had lower bursa/bodyweight ratios compared to uninfected controls. The difference was more apparent towards the end of the study period. In addition to acting as a main target tissue for the cytolytic phase of MDV, the bursa may also be the site for the next phase of the MDV life cycle, which is infection of T cells. T cells from CD4, CD8 or double positive T cells (CD4+ and CD8+) subsets may be recruited to the site of infection via various mechanisms. It has been demonstrated that MDV-infected cells up-regulate MHC class II molecules in the bursa of Fabricius (Niikura et al., 2007), facilitating antigen presentation (Malnati et al., 1992) and subsequent stimulation and activation of T cells. This may indeed be one of the triggers for cellular infiltration and cytokine production observed in the present study. However, the role of MHC class I in response to MDV infection and activation of T cells may be less clear, because it has been shown to be down-regulated after MDV infection (Gimeno et al., 2001; Hunt et al., 2001; Levy et al., 2003). In addition to MHC class II, cytokines and chemokines produced by other infiltrating cells or vIL-8 produced by MDV, may play a role in T cell recruitment and activation and, ultimately, facilitating the switch of infection from B cells to T cells (Liu et al. 1999; Parcells et al. 2001; Cui et al. 2004). In agreement with this view, in the present study, vIL-8 expression was at its highest at 4 d.p.i. Using recombinant MDV lacking vIL-8, it has been shown that vIL-8 expression is essential for the cytolytic phase in the bursa of Fabricus (Cui et al. 2004). Furthermore, Heidari et al. (2008) have shown that the expression of vIL-8 gene is significantly higher at 5 d.p.i. compared to 15 d.p.i.

In the present study, the early infiltration of macrophages as well as CD4+ and CD8+ T cells was confined mainly to inter-follicular areas which are known as T-dependent areas (Cortes et al., 1995) and responsible for the function of the bursa of Fabricius as a secondary lymphoid organ (Ekino et al., 1985). During the observation period, macrophages and T cells had infiltrated into the affected follicles. In this environment, MDV could perhaps switch from B cells to activated T cells. At the time when host responses are initiated, MDV establishes latency in T cells, particularly in CD4+ T cells, that have infiltrated the infected tissue. Although the majority of CD4+ and CD8+ cells are expected to be single positive T cells, there may also be a small population of double positive T cells and non-T cell populations expressing these molecules.

Our observation that the expression of pp38 antigen as well as gB mRNA and vIL-8 mRNA was reduced between 4–7 d.p.i. may be an indication of the start of latency. During the cytolytic phase, MDV proteins such as pp38, gB and vIL-8 play an important role in viral pathogenesis, while their expression is reduced during the latency phase (Ross et al. 1997; Baigent et al., 1998; Burgess et al., 2001; Burgess and Davison, 2002; Reddy et al., 2002; Cui et al. 2004; Gimeno et al., 2005; Heidari et al., 2008). Among MDV genes, meq has been shown to be expressed during the latent phase and may be important for the maintenance of this phase (Kung et al., 2001; Heidari et al., 2008). In agreement with these results, we observed a consistent up-regulation in meq expression throughout the study period. This also coincided with an increase in MDV genome load. There are two

scenarios to explain why MDV genome load had significantly increased at 14 d.p.i. It is possible that by 14 d.p.i. some of the latent viruses had reactivated to begin the second cytolytic phase of their replication cycle (Calnek, 2001). Secondly, some latent viruses may undergo replication, similar to what has been observed for other lymphotropic herpesviruses, such as Epstein-Barr virus (EBV). Latent EBV may undergo replication for the purpose of maintaining viral genome in dividing lymphocytes (Babcock et al., 1999). Further studies are required to test these scenarios in the case of MDV.

Avian macrophages play an important role as part of the innate immune system by producing cytokines and exerting phagocytic functions (Qureshi et al., 2000). In natural MDV infection, infected macrophages associated with the respiratory system are suggested to carry MDV from the site of initial infection to the bursa of Fabricius (Barrow et al., 2003). In the present study, an association was observed between MDV infection in the bursa and infiltration of macrophages during the early post-infection period. Macrophage infiltration in response to MDV infection has been recorded in the brain early following infection (Gimeno et al., 2001). In agreement with the present finding, an association has been observed between IBDV infection in the bursa and early infiltration of macrophages (Withers et al., 2005; Khatri et al., 2005; Rautenschlein et al., 2007). Our study did not elucidate the exact role played by macrophages in the bursa after infection, but it is possible that these cells were involved in the expression of some of the cytokines that were measured. Others have shown that in IBDV infection, the infiltrating macrophages may express proinflammatory cytokines (Khatri et al., 2005; Palmquist et al., 2006), such as IL-18. Similarly in the present study, IL-18 was marginally higher in the bursa of MDV-infected chickens at 4 d.p.i. when infiltration of macrophages into the bursa was at its highest. The importance of macrophages in MDV pathogenesis is several fold. Peritoneal macrophages have been shown to inhibit MDV replication in vitro (Kodama et al., 1979). Thus, it is possible that macrophages in the present study were involved in inhibition of MDV replication. Secondly, activated macrophages are known to be a source of IFN-γ (Frucht et al., 2001) and in the present study, IFN-γ was up-regulated in the bursa of MDV-infected chickens at early time points post-infection. The direct inhibitory effect of IFN-γ on MDV has been shown in vitro (Levy et al., 1999). It is also possible that macrophages may have played a role in clearing MDV-infected cells by phagocytosis (Powell et al., 1983; Djeraba et al., 2000). Since in the present study the expression of iNOS was significantly higher in the bursa of MDV-infected chickens and correlated significantly with macrophage counts in the bursa, macrophages may have curtailed MDV replication through NO production as has been described previously (Lee, 1979; Djeraba et al., 2000; Xing and Schat, 2000b).

The role of T cell subsets, particularly CD8+ T cells in response to MDV infection has been previously studied (Morimura et al., 1999; Schat and Xing, 2000; Garcia-Camacho et al. 2003). An association between tissue infiltration of CD4+ and CD8+ T cells and response to MDV has been described in brain (Gimeno et al., 2001) and feathers (Abdul-Careem et al., 2008). The present study also provides evidence that both CD4+ and CD8+ T cells are associated with MDV infection in the bursa. Similar to our observations, accumulation of CD4+ and CD8+ T cells has also been reported in the bursa of IBDV-infected chickens (Tanimura and Sharma, 1997; Kim et al., 1999; Kim et al., 2000) and depletion of these two subsets of T cells has been shown to increase IBDV viral load in the bursa (Kim et al., 2000). Since CD8+ cytotoxic cells have been demonstrated to effectively reduce virus replication in MDV-infected chickens (Morimura et al., 1999), CD8+ T cells may have a role in clearing virally infected cells in the bursa as well. This notion is supported by the observation that there was a surge in CD8+ T cell infiltration in the bursa coinciding with a reduction in MDV gB transcripts and the number of cells positive for viral antigens. In addition to CD8+ T cells, CD4+ T cells were also

present in the bursa of MDV-infected chickens. It is likely that at least some of the CD4+ T in the tissue were involved in direct or indirect antiviral immune responses. These T cell subsets may serve as a source of cytokines such as IFN-γ (Göbel et al., 2003; Ngai et al., 2007), which was highly up-regulated after MDV infection in our study. However, the possibility that some of the T cells infiltrating the tissue, especially at 7, 10 and 14 d.p.i. were in fact cells which were latently infected (Buscaglia et al., 1988) with MDV cannot be ruled out.

Of the cytokines that were studied, only IFN-α and IFN-γ were significantly up-regulated in the bursa of infected birds. Type I interferons, such as IFN-α, are well known to play a role in clearing herpesvirus-infected cells (Mossman and Ashkar, 2005). In relation to MDV infection, the expression of IFN-α is influenced by the genetic background of chickens (Quere et al., 2005). In MD-resistant chickens, the expression of IFN-α has been shown to be down-regulated (Xing and Schat, 2000a). In the present study, up-regulation of IFN-α gene was observed in response to very virulent MDV infection in the bursa. The association between the expression of IFN-γ gene and MDV infection has been well documented (Xing and Schat, 2000a; Kaiser et al., 2003; Jarosinski et al., 2005; Abdul-Careem et al., 2007, 2008). However, the expression of this cytokine in the bursa in response to MDV had not been previously studied. Others, however, have reported the expression of this cytokine in the bursa and its significance in response to IBDV infection (Kim et al., 2000; Rautenschlein et al., 2003; Eldaghayes et al., 2006; Rautenschlein et al., 2007). Here, we also report an increase in the expression of IFN-γ gene in response to MDV replication in the bursa. The up-regulation of IFN-γ along with significant infiltration of macrophages and T cell subsets suggests an important role for this cytokine in cell-mediated immune response against MDV infection in the bursa. The observed IFN-γ response in the bursa may be involved in direct antiviral activity against MDV as it has been previously shown that IFN-γ can elicit direct antiviral activity against the RB1B and CVI988 strains of MDV as well as herpesvirus of turkeys (HVT) in chicken embryo fibroblast cell cultures (Levy et al., 1999, 2003). In addition, it is possible that in our study, IFN-γ may have affected MDV replication through macrophage activation followed by NO production by these cells in the bursa of infected chickens (Lee, 1979; Djeraba et al., 2000; Xing and Schat, 2000b). Indirectly, IFN-γ might have up-regulated the expression of major histocompatibility complex (MHC) class II molecules in virus-infected cells in the bursa (Niikura et al., 2007), thus enhancing antigen presentation leading to induction or intensification of immune response (Guidotti and Chisari, 2001).

In conclusion, MDV replication in the bursa of Fabricius is associated with an early up-regulation of cytokine genes such as IFN-α and IFN-γ and infiltration of cells such as macrophages, CD4+ and CD8+ T cells. The results suggest a role for these cells and cytokines in MDV-induced host responses in bursa of Fabricius. Further investigations should shed more light on the function of these cells and the cytokine they produce in viral pathogenesis and immunity against MDV.

Materials and methods

Virus strain

Chickens were infected with MDV strain RB1B (passage 9) that was provided by Dr. K.A. Schat (Cornell University, NY, USA) (Schat et al., 1982).

Experimental animals

Day-old specific pathogen free (SPF) White Leghorn chickens were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario, Canada). Chicks were housed in isolation facility of Ontario Veterinary College. The chickens were free of maternal antibodies against MDV and the B haplotypes were not known.

Experimental design

Thirty-six day-old chicks were randomly divided into two groups. Twenty chickens were infected intraperitoneally on day 5 of age with 250 plaque-forming units (PFU) of the RB1B strain of very virulent MDV. The rest ($n=16$) were kept as uninfected controls. At 4, 7, 10 and 14 d.p.i. five MDV-infected chickens were euthanized by CO_2 inhalation and necropsied along with four uninfected controls. Before being examined, the chickens were weighed. At necropsy, the bursa of Fabricius was collected from each bird and weighed. Bursa samples were collected from three chickens in each of the infected and uninfected control groups. Samples were then snap frozen in embedding medium (Tissue-Tek, Sakura Fine tek USA, Inc. Torrance, CA, USA) for immunohistochemical analysis. For RNA and DNA extraction, all chickens in each of the infected and uninfected groups were sampled and bursa samples were stored in RNAlater (Qiagen Inc., Mississauga, Ontario, Canada) at −20 °C.

DNA and RNA extraction

DNA and RNA extraction from the bursa of Fabricius collected at 4, 7, 10 and 14 d.p.i. was carried out using Trizol (Invitrogen Canada Inc., Burlington, Ontario, Canada) as has been described previously (Abdul-Careem et al., 2006b).

Reverse transcription

Reverse transcription of total RNA (2 μg) was carried out using Oligo(dT)$_{12-18}$ primers (SuperScript™ First–Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions.

Primers

The absolute MDV genome load in the bursa of Fabricius were quantified using primers specific for the meq gene of MDV (Abdul-Careem et al., 2006a). The same pair of primers was used for the relative quantification of MDV meq expression in cDNA preparations derived from the bursa of Fabricius. The previously published primers were used for the relative quantification of expression of target genes (iNOS, MDV gB, IL-10, IL-18, IFN-α, IFN-γ) and β-actin that acted as the reference gene (Abdul-Careem et al., 2006b, 2008; Brisbin et al., 2007). The primers specific for chicken IFN-β gene were designed to span across exon and intron boundaries after alignment of the relevant nucleotide sequences in the GenBank database (accession No. X92479) using the Vector NTI™ software (Version 5.5, InforMax, Inc., Frederick, Maryland, USA). IFN-β primers were as follows: F-5′-GCCTCCAGCTCCTTCAGAATACG-3′, R-5′-CTGGATCTGGTTGAG-GAGGCTGT-3′. The primers specific for vIL-8 gene were designed after alignment of the relevant nucleotide sequences in the GenBank database (accession No. AF489276) using the Vector NTI™ software (Version 5.5, InforMax, Inc., Frederick, Maryland, USA). vIL-8 primers were as follows: F-5′-TATCACTGGAGAGTCTCGCTGTC-3′, R-5′-TCAGTCCTCCTGCAGTGTATACC-3′. The primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Conventional PCR

Conventional PCR for the detection of MDV meq gene in the bursa of Fabricius DNA preparations was done for initial screening as has been described previously (Abdul-Careem et al., 2006a).

Preparation of constructs as standards

Real-time PCR and RT-PCR quantification of MDV genome load and gene expression, respectively, were done using standard curves. The standard curves for MDV meq gene, IL-10, IL-18, IFN-γ, β-actin and iNOS genes have been described previously (Abdul-Careem et al., 2006a,b, 2007). Standard curves for IFN-α, IFN-β and vIL-8 genes were generated in duplicate using the same protocol used for the other genes as described previously (Abdul-Careem et al., 2006a). All PCR-amplified products were cloned into pDrive (QIAGEN® PCR Cloning Kit, QIAGEN Inc., Ontario). For construction of standard curves of target and reference genes, 10-fold serial dilutions (10^{-1} to 10^{-9}) of the relevant plasmid DNA preparations were made and assayed in duplicate.

Real-time PCR and RT-PCR

All the samples were run in simplex since within assay variability for all the assays had a coefficient variation of <5% (data not shown). All the cDNA preparations were tested in a single real-time RT-PCR assay along with a dilution series of the standard that served as the calibrator and a no template control. All the real-time RT-PCR runs were conducted in a LightCycler® 480 multiwell plate 384 (Roche Diagnostics GmbH, Mannheim, State of Baden-Wurttemberg, Germany) in a final volume of 20 μl of LightCycler ® 480 SYBR Green 1 (Master LightCycler FastStart DNA Master SYBR Green 1 was used for the amplification of IFN-γ) (Roche Diagnostics GmbH) containing fast start Taq DNA polymerase for 'hot start' and DNA intercalating dye SYBR Green 1 for detection in a LightCycler® 480 thermocycler (Roche Diagnostics GmbH). The real-time PCR assays for the quantification of MDV meq and vIL-8 genes were conducted in glass capillaries (Roche Diagnostics GmbH, Mannheim, State of Baden-Wurttemberg, Germany) in a final volume of 20 μl of LightCycler FastStart DNA Master SYBR Green 1 (Roche Diagnostics GmbH, Mannheim, State of Baden-Wurttemberg, Germany) containing fast start Taq DNA polymerase for 'hot start' and DNA intercalated dye SYBR Green 1 dye for detection in a LightCycler thermocycler, version 3.5 (Roche Diagnostics GmbH, Mannheim, State of Baden-Wurttemberg, Germany). In addition, the reaction consisted of 0.25–0.5 μM of each of the gene-specific primers and 5 μl of 1:10 dilution of cDNA as template (2 μl was used for the amplification of IFN-γ, MDV meq, MDV gB and vIL-8 genes) and PCR grade water.

The optimum thermal cycling parameters varied according to the gene and included pre-incubation at 95 °C for 10 min; 40–45 cycles of amplification at 95 °C for 10 s, 64 °C for 5 s (55 °C/5 s for the amplification of IL-10 gene) and 72 °C for 10 s (72 °C/5 s, 72 °C/15 s and 72 °C/7 s for the amplification of IL-10, IFN-γ and MDV meq and gB genes, respectively); melting curve analysis at 95 °C for 5 s (95 °C for 1 s for IL-6) (segment 1), 65 °C/1 min (segment 2) and 97 °C/0 s (segment 3); cooling at 40 °C/10 s. Fluorescent acquisition was done at 72 °C/10 s (72 °C/5 s and 72 °C/7 s for the amplification of IL-10, IFN-γ and MDV meq and gB genes, respectively).

Histological observation

The bursa of Fabricius samples preserved in embedding medium for frozen tissue specimens were sectioned (thickness 5 μm) using a cryotome (LEICA CM 3050 S, Vashaw Scientific Inc., Norcross, Atlanta, GA, USA), adhered to microslides (Superfrost plus, VWR Labshop, Betavia, IL, USA) and preserved at −20 °C until used. The immunohistochemistry technique, as described by Abdul-Careem and colleagues (2008), was used to assess the distribution of MDV pp38 positive cells, monocyte/macrophages, and CD4+/CD8+ T cells in serial sections of the bursa in order observe the same follicle for different cell subsets and pp38+ cells. The monoclonal antibody against MDV pp38 antigen was provided by the Avian Disease and Oncology Laboratory, U.S. Department of Agriculture, East Lansing, MI, USA and used at 1:2000 dilution in blocking buffer. Monoclonal antibodies specific for chicken CD4 (clone CT-4) and CD8a (clone CT-8) that have been raised in mice (Southern Biotech, Birmingham, Alabama, USA) were used at 1:200 dilution in blocking buffer. Monoclonal antibody KUL01 specific for mononuclear phagocytes in chicken that has been raised in mice was used (Southern Biotech, Birmingham, Alabama, USA) in 1:400 dilution in blocking buffer. Avidin-biotin-peroxidase complex (ABC) system (Vectastain® ABC kit, Vector Laboratories, Burlingame, CA, USA) was used for immunoperoxidase staining of tissue sections according to the manufacturer's instructions. Quenching of the endogenous peroxidase activity was done by treating the sections for 10–20 min with 3% hydrogen peroxide with 0.3% goat serum made in phosphate buffered saline (PBS). A 5% goat serum in PBS was used as the blocking agent in all immunohistochamical studies. Biotinylated goat anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. Samples were incubated with the primary antibodies for 30 min at room temperature followed by rinsing and incubation with the secondary antibody for 30 min in a humidified chamber. Antigen localization was visualized by incubation of the sections with 3,3-diaminobenzidine-H_2O_2 solution (DAB substrate kit for peroxidase, Vector Laboratories, Burlingame, CA, USA). The slides were counter stained with hematoxyline (Protocol, Fisher Scientific Company, Kalamazoo, MI, USA) and mounted in Cytoseal-60 (Richard-Allan-Scientific, Kalamazoo, MI, USA).

Three animals from each time point and each group (MDV-infected and -uninfected control) were examined in order to characterize the pattern of infiltration of macrophages as well as CD4+ and CD8+ T cells and MDV pp38 + cells. The degree of infiltration of each type of cells in the bursa of Fabricius over time was assessed quantitatively. Briefly, 5 highly infiltrated fields of 40x magnification were chosen from each section and immunoperoxidase-stained cells were enumerated.

Data analysis

Bursa weights are expressed as a ratio of bodyweight. Cells counted in five fields of 40x magnification for each chicken were averaged and subjected to statistical analysis. Quantification of MDV genome load and expression of cytokine genes by real-time PCR and RT-PCR was done as has been described previously (Abdul-Careem et al., 2006a,b). Briefly, the absolute number of MDV genomes per 100 ng of DNA of the bursa of Fabricius samples was calculated based on an external standard curve. The expression of cytokine genes was calculated relative to the expression of β-actin gene and expressed as ratios. Correlation between macrophage counts and the expression of iNOS gene was analyzed using the statistical package, MINITAB® release 14 (Minitab Inc., State College, Pennsylvania, USA). Bursa/bodyweight ratios, real-time PCR data and cell counts were subjected to t test (GraphPad software Inc., CA 92037 USA) in order to identify differences between groups. Before being tested, each set of data was analyzed using the Grubbs' test (GraphPad software Inc., CA 92037 USA) to identify outliers. Comparisons were considered significant at $P \leq 0.05$.

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